No association of multiple sclerosis activity and progression with EBV or tobacco use in BENEFIT

Kassandra L. Munger, ScD
Kathryn C. Fitzgerald, MSc
Mark S. Freedman, MD
Hans-Peter Hartung, MD
David H. Miller, MD
Xavier Montalbán, MD
Gilles Edan, MD
Frederik Barkhof, MD
Gustavo Suarez, MD
Ernst-Wilhelm Radue, MD
Rupert Sandbrink, MD
Ludwig Kappos, MD
Christoph Pohl, MD†
Alberto Ascherio, MD, DrPH

Correspondence to
Dr. Munger:
kgorham@hsph.harvard.edu

ABSTRACT

Objective: To evaluate whether Epstein-Barr virus (EBV) immunoglobulin G (IgG) antibody levels or tobacco use were associated with conversion to multiple sclerosis (MS) or MS progression/activity in patients presenting with clinically isolated syndrome (CIS).

Methods: In this prospective, longitudinal study, we measured EBV IgG antibody and cotinine (bio-marker of tobacco use) levels at up to 4 time points (baseline, months 6, 12, and 24) among 468 participants with CIS enrolled in the BENEFIT (Betaferon/Betaseron in Newly Emerging Multiple Sclerosis for Initial Treatment) clinical trial. Outcomes included time to conversion to clinically definite or McDonald MS, number of relapses, Expanded Disability Status Scale (EDSS) changes, brain and T2 lesion volume changes, and number of new active lesions over 5 years. Analyses were adjusted for age, sex, treatment allocation, baseline serum 25-hydroxyvitamin D level, number of T2 lesions, body mass index, EDSS, steroid treatment, and CIS onset type.

Results: We found no associations between any EBV IgG antibody or cotinine levels with conversion from CIS to MS or MS progression as measured by EDSS or activity clinically or on MRI. The relative risk of conversion from CIS to clinically definite MS was 1.14 (95% confidence interval 0.76–1.72) for the highest vs the lowest quintile of EBNA-1 IgG levels, and 0.96 (95% confidence interval 0.71–1.31) for cotinine levels >25 ng/mL vs <10.

Conclusions: Neither increased levels of EBV IgG antibodies, including against EBNA-1, nor elevated cotinine levels indicative of tobacco use, were associated with an increased risk of CIS conversion to MS, or MS activity or progression over a 5-year follow-up. *Neurology*® 2015;85:1694–1701

GLOSSARY

BENEFIT = Betaferon/Betaseron in Newly Emerging Multiple Sclerosis for Initial Treatment; **CDMS** = clinically definite multiple sclerosis; **CIS** = clinically isolated syndrome; **CMV** = cytomegalovirus; **CV** = coefficient of variation; **EBNA-1** = Epstein-Barr virus nuclear antigen-1; **EBV** = Epstein-Barr virus; **EDSS** = Expanded Disability Status Scale; **IgG** = immunoglobulin G; **MDMS** = McDonald multiple sclerosis; **MS** = multiple sclerosis; **RR** = relative risk; **VCA** = viral capsid antigen.

Elevated immunoglobulin G (IgG) antibodies against Epstein-Barr virus nuclear antigen-1 (EBNA-1) and cigarette smoking are established risk factors for multiple sclerosis (MS).¹ However, there is less understanding of whether and how they are involved in MS disease activity and progression.

Results of previous studies on Epstein-Barr virus (EBV) IgG antibody titers, cigarette smoking, and MS activity and progression have been mixed. Three prior studies examining EBV and MS activity and/or progression among patients with clinically isolated syndrome (CIS) (first neurologic event suggestive of MS) suggest there is no association with conversion to MS, but have shown mixed results regarding activity and progression.^{2–4} Two studies on the effects of smoking and CIS report conflicting results regarding the association between smoking and conversion to clinically definite MS.^{2,5}

In this study, we examined whether serum levels of anti-EBV IgG antibodies or cotinine, a marker of tobacco use, are associated with early MS disease activity and progression among

†Deceased.

From the Departments of Nutrition (K.L.M., K.C.F., A.A.) and Epidemiology (A.A.), Harvard T.H. Chan School of Public Health, Boston, MA; Ottawa Hospital Research Institute (M.S.F.), Canada; Heinrich-Heine Universität (H.-P.H., R.S.), Düsseldorf, Germany; UCL Institute of Neurology (D.H.M.), London, UK; Hospital Universitari Vall d'Hebron (X.M.), Barcelona, Spain; CHU-Hôpital Pontchaillou (G.E.), Rennes, France; VU University Medical Center (F.B.), Amsterdam, the Netherlands; Bayer HealthCare Pharmaceuticals (G.S.), Montville, NJ; Medical Image Analysis Center (E.-W.R.), and Neurology, Departments of Medicine, Clinical Research and Biomedicine (L.K., C.P.), University Hospital Basel, Switzerland; Bayer HealthCare (R.S.), Berlin; and Department of Neurology (C.P.), University Hospital of Bonn, Germany.

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participants in the Betaferon/Betaseron in Newly Emerging Multiple Sclerosis for Initial Treatment (BENEFIT) clinical trial, a cohort of patients who were enrolled and randomized to receive either interferon beta-1b or placebo soon after experiencing a CIS.

METHODS **Standard protocol approvals, registrations, and patient consents.** Participants in the BENEFIT clinical trial (NCT00185211) provided written informed consent, and this study was approved by the Harvard School of Public Health's institutional review board. This study was completed using de-identified data and serum samples.

Study population. The BENEFIT trial included 468 participants who were randomized to receive either interferon beta-1b or placebo within 60 days of experiencing a CIS, and followed for conversion to MS (both clinically definite MS [CDMS] and McDonald MS [MDMS]). After experiencing CDMS or after 2 years, placebo patients were switched to interferon beta-1b treatment. All patients had been followed for a total of 5 years. Detailed information on the trial and its participants are provided elsewhere.⁶ Relapses were recorded as they occurred, Expanded Disability Status Scale (EDSS) was assessed, and MRIs were conducted at baseline, then every 3 months through month 12, and then at months 18, 24, 36, 48, and 60. EDSS was additionally assessed at months 30, 42, and 54. Serum samples were obtained at baseline (before randomization to treatment) and at 6, 12, and 24 months, were received by the central laboratory within 3 days of collection, and stored at -20°C ; they were first thawed and aliquoted for this study. Aliquots were then refrozen and shipped to the analyzing laboratory.

MRI. The MRI procedures used in BENEFIT have been previously described.^{6,7} T2- and T1-weighted images after administration of 0.1 mmol/kg of gadolinium–diethylenetriaminepentaacetic acid were read at the Image Analysis Centre at the VU University Medical Center (Amsterdam), and number of new lesions, newly active lesions, and lesion volume were determined. Brain volume was measured using the SIENAX (structural image evaluation using normalization of atrophy cross-sectional) method.

Viral serology. All viral IgG antibodies were measured using ELISA (DiaMedix Corp., Miami, FL). IgG antibodies to EBNA-1 were measured in all available serum samples. EBV viral capsid antigen (VCA) IgG and cytomegalovirus (CMV) IgG were measured in the 6- and 12-month samples. For the EBV antibodies, an ELISA index value <0.90 was considered negative, 0.90 – 1.09 equivocal, and ≥ 1.10 positive, per the manufacturer's instructions. Similarly for CMV, an index value <8.0 was negative, 8.0 – 9.99 equivocal, and ≥ 10 positive. Quality-control samples were included among the study samples. Intra-assay coefficients of variation (CVs) ranged from 0.95% (EBNA-1) to 6.7% (CMV), and interassay CVs ranged from 4.2% (EBNA-1) to 14.8% (CMV).

Cotinine. Cotinine is a nicotine metabolite and is a reliable biomarker of recent/current tobacco use.⁸ Cotinine was measured in the baseline and 6-, 12-, and 24-month samples using an ELISA (DiaMedix Corp.). Cotinine levels >25 ng/mL are indicative of recent tobacco use, while nonusers generally have levels <10 ng/mL. The CVs calculated from quality-control samples were 3.1% to 7.2% for intra-assay and 12.7% to 13.4% for interassay.

Statistical analysis. There were 3 outcomes of interest: (1) time to conversion from CIS to MS (either CDMS or MDMS); (2)

relapse rates and changes in EDSS score; and (3) changes in number of active lesions, volume of T2 lesions, and brain volume. To minimize reverse causation and changes possibly due to resolution of the CIS, month 6 was considered baseline in analyses of clinical and MRI outcomes, except for change in T2 lesion volume and brain volume in which analyses at month 12 were considered baseline because volume measures in the first year may be affected by the acute inflammation of CIS (e.g., resolution of edema or pseudoatrophy).^{7,9} The earliest VCA and CMV IgG levels were measured at month 6; therefore, in analyses of these exposures, time to conversion to MS began at month 6.

Viral antibody index values were modeled either as continuous variables or categorically as quartiles that were derived from the antibody distribution at each specific time point. In cotinine analyses, we considered all cotinine measures (up to 4) through 24 months as a proxy for smoking status. If cotinine was missing for a particular time point, we carried forward the previous measurement. We used the 6-month cotinine level as baseline for 14 patients missing baseline cotinine levels. Three cotinine categories were created: cotinine levels >25 ng/mL in all available samples (indicative of consistent tobacco use), cotinine <10 ng/mL in all available samples (indicative of no tobacco use), and cotinine levels both above and below the a priori–defined levels (indicative of change in tobacco use). We also created a group with high tobacco use defined as having average cotinine levels above the median level (190 ng/mL) in all those with levels >25 ng/mL. In analyses using baseline-only cotinine levels (including only those with nonmissing baseline values), these cutpoints were also used to divide participants into 3 groups. Cox proportional hazards models were used to estimate the hazard ratio of conversion from CIS to MS from baseline to month 60 (EBNA-1 IgG, cotinine) and from month 6 to month 60 (EBNA-1, VCA, and CMV IgG). We used generalized linear mixed models accounting for the repeated within-person measures to assess the associations between viral IgG antibody or cotinine levels and annualized relapse rate, change in EDSS score, and percent change in brain and T2 lesion volume. Changes in EDSS and MRI measures were determined with respect to either the 6-month (EDSS) or 12-month (brain and T2 lesion volume) values. For example, for each EDSS time point (up to 11 per individual), we calculated the difference between the EDSS score at that time point and the one at 6 months; these differences were modeled to estimate the slope of EDSS change. This approach minimizes the influence of spurious short-term changes in the EDSS/MRI volume measures. Negative binomial mixed models were used to assess the associations with number of new active lesions.

Analyses were adjusted for age, sex, treatment allocation (interferon beta-1b or placebo), baseline serum 25-hydroxyvitamin D level, number of T2 lesions at baseline, body mass index at baseline, EDSS score at baseline, steroid treatment at baseline, and CIS onset type (monofocal or multifocal). Cotinine analyses were also stratified by sex because previous studies have suggested that the risk of MS associated with smoking may be different in men and women.^{10–12} All analyses were also conducted stratified by initial treatment allocation (placebo or interferon beta-1b).

RESULTS Of the 457 participants with an available baseline sample, all but 9 (2%) were seropositive for EBNA-1 IgG. Among these 9, 6 had undetectable and 3 equivocal EBNA-1 IgG index values. Of the 6 EBV-negative participants, 4 showed evidence of EBV infection in a later sample and 2 of them developed MS; one remained EBV negative and did

Table 1 Baseline characteristics by baseline EBNA-1 IgG quartile and cotinine levels

	Baseline EBNA-1 quartile ^a				Cotinine ^b	
	1	2	3	4	<10 ng/mL	>25 ng/mL
Median (range)	3.91 (1.16–4.16)	4.32 (4.16–4.44)	4.58 (4.44–4.71)	4.87 (4.71–5.32)	2.6 (1.6–9.0)	164 (25.3–399)
No. (%)	113	111	112	112	278 (60)	173 (37)
Age at baseline, median (Q1–Q3), y	31 (25–36)	30 (24–37)	29 (23–37)	30 (24–36)	31 (25–38)	29 (24–34)
Female, %	78.8	73.9	65.2	67	77	63
IFNβ-1b treatment group, %	55.8	61.3	70.5	61.6	63	61
Monofocal onset, %	45.1	48.7	57.1	58	52	52
T2 lesion volume at screening, median (Q1–Q3), mm ³	1,974 (621–4,890)	1,903 (956–5,247)	1,731 (521–4,602)	2,047 (624–4,219)	1,758 (621–4,249)	2,079 (592–5,215)
No. of T2 lesions at screening, median (Q1–Q3)	20 (7–43)	19 (9–41)	16 (7.5–36)	17 (6–34.5)	18 (7–37)	16 (7–39)
Central brain volume at screening, median (Q1–Q3), cm ³	1,059 (1,021–1,084)	1,057 (1,016–1,081)	1,057 (1,027–1,091)	1,054 (1,034–1,084)	1,056 (1,022–1,084)	1,056 (1,029–1,087)
EDSS score at baseline, median (Q1–Q3)	1.5 (1.0–2.0)	1.5 (1.0–2.0)	1.5 (1.0–2.0)	1.5 (1.0–2.0)	1.5 (1.0–2.0)	1.5 (1.0–2.0)
BMI, median (Q1–Q3), kg/m ²	22.8 (21.2–25.4)	22.9 (20.5–26)	23.4 (20.9–27)	24.3 (21.6–27.8)	23.2 (21–26.3)	23.5 (21.3–27.4)
25(OH)D at baseline, median (Q1–Q3), nmol/L	44.9 (34.4–61.6)	45.8 (32.4–54.6)	41.7 (31–54.2)	43.5 (30.9–56.5)	44.8 (32.3–59.1)	41.2 (31.5–54.7)
Steroid use, %	74.3	74.8	73.2	62.5	71	70

Abbreviations: 25(OH)D = 25-hydroxyvitamin D; BMI = body mass index; EBNA-1 = Epstein-Barr virus nuclear antigen-1; EDSS = Expanded Disability Status Scale; IFNβ-1b = interferon beta-1b; IgG = immunoglobulin G; Q1–Q3 = quartile 1–quartile 3.

^a Among EBNA-1-positive individuals.

^b Active smoker >25 ng/mL.

not develop MS, and one had only the negative baseline sample available but also did not develop MS during the study.

Baseline analyses were restricted to the 448 individuals positive for EBNA-1 IgG. Demographic, clinical, and MRI characteristics were similar across quartiles of baseline EBNA-1 index values, except for an increasing percentage of participants with a monofocal onset with increasing baseline EBNA-1 IgG levels (table 1). Those with the highest levels of EBNA-1 IgG antibody also had a higher baseline body mass index and were less likely to be treated with steroids at onset (table 1).

Men were more likely to have cotinine levels >25 ng/mL ($p = 0.002$); otherwise, baseline characteristics were evenly distributed between cotinine groups (table 1). Cotinine levels were relatively stable over the 24 months of measurements. Of the 173 individuals with cotinine levels >25 ng/mL at baseline, 149 (86%) continued to have cotinine levels >25 ng/mL in all available samples. Similarly, of the 278 individuals with cotinine levels <10 ng/mL, 253 (91%) remained <10 ng/mL in all the remaining available samples. There were 55 cases with cotinine levels not consistently <10 ng/mL or >25 ng/mL; 24 had levels >25 ng/mL and 25 had levels <10 ng/mL at baseline; and 6 had equivocal baseline levels >10 but <25 ng/mL.

CIS conversion to MS. Individuals in the top vs bottom quartile of baseline EBNA-1 IgG converted from CIS to CDMS or MDMS at similar rates (figure, A; table 2). Starting the follow-up at 6 months post baseline, levels of IgG at 6 months to EBNA-1, VCA (figure, B), or CMV (figure, C) also did not predict conversion to CDMS or MDMS through year 5 (table 2). Because of smaller sample sizes, we also examined risk of conversion in those with VCA or CMV IgG levels above vs below the median levels and there was still no association: VCA, median IgG = 4.22, relative risk (RR)_{CDMS} = 1.22 (95% confidence interval [CI] 0.86–1.72), RR_{MDMS} = 1.15 (95% CI 0.84–1.56); and CMV, median IgG = 140, RR_{CDMS} = 0.78 (95% CI 0.48–1.27), RR_{MDMS} = 0.90 (95% CI 0.60–1.36).

Neither baseline nor average cotinine levels >25 ng/mL or >190 ng/mL were associated with an increased hazard rate of conversion to CDMS or MDMS from baseline to month 60, as compared with levels <10 ng/mL (figure, D; table 2); these results did not differ by sex.

Relapse rate and change in EDSS score. There was no association between levels of either EBV or CMV IgG antibody at 6 months or cotinine levels and relapse rate through year 5 (table 3). None of the viral IgG antibodies were associated with changes in EDSS

Kaplan-Meier curves for risk of conversion to CDMS by (A) quintiles of EBNA-1 IgG antibody, (B) quintiles of VCA IgG antibody, (C) quintiles of CMV IgG antibody, and (D) tobacco use (cotinine levels >25 ng/mL indicates use vs <10 ng/mL indicates no use). Log-rank p values: baseline EBNA-1: $p_{\text{log-rank}} = 0.10$; 6-month VCA: $p_{\text{log-rank}} = 0.39$; 6-month CMV: $p_{\text{log-rank}} = 0.33$; cotinine: $p_{\text{log-rank}} = 0.75$. CDMS = clinically definite multiple sclerosis; CMV = cytomegalovirus; EBNA-1 = Epstein-Barr virus nuclear antigen-1; EBV = Epstein-Barr virus; IgG = immunoglobulin G; VCA = viral capsid antigen.

score from month 6 through 60 (EBNA-1: multivariate adjusted $\beta = 0.02$, 95% CI -0.10 to 0.13 , $p = 0.80$; VCA: $\beta = -0.003$, 95% CI -0.10 to 0.10 , $p = 0.95$; CMV: $\beta = -0.001$, 95% CI -0.003 to 0.001 , $p = 0.25$). Individuals with cotinine levels >25 ng/mL or >190 ng/mL also did not have a statistically significant greater change in EDSS score from month 6 through 60 than those with cotinine levels <10 ng/mL (>25 ng/mL: $\beta = 0.10$, 95% CI -0.06 to 0.25 , $p = 0.22$; >190 ng/mL: $\beta = 0.03$, 95% CI -0.16 to 0.23 , $p = 0.73$). There were no differences in the associations between cotinine levels and EDSS score by sex.

MRI measures. Neither the viral IgG levels nor cotinine level was associated with the percent changes

in T2 lesion volume or brain volume from month 12 through month 60. Similarly, there were no associations between any of the viral IgG levels or cotinine levels and rate of cumulative newly active lesion development (table 4). There were no statistically significant associations between cotinine levels and MRI measures by sex.

All of the above analyses were also conducted separately by randomized treatment group (placebo or interferon beta-1b) and no associations between EBV, CMV, or cotinine and any of the outcomes were observed (data not shown).

DISCUSSION In this large prospective study among patients with CIS, neither higher EBNA-1 IgG antibody levels nor cotinine concentrations were

Table 2 Hazard ratio of conversion to CDMS/MDMS by EBV IgG antibody and cotinine levels

	CDMS			MDMS		
	No.	RR ^a	95% CI	No.	RR ^a	95% CI
Baseline EBNA-1						
Q1	113	1	Ref.	113	1	Ref.
Q2	111	1.38	0.94-2.02	111	1.46	1.09-1.95
Q3	112	1.18	0.79-1.77	112	1.08	0.80-1.45
Q4	112	1.14	0.76-1.72	112	1.04	0.78-1.40
6-mo^b VCA						
Q1	74	1	Ref.	59	1	Ref.
Q2	78	1.18	0.69-2.02	57	1.08	0.68-1.71
Q3	86	1.54	0.93-2.55	58	1.29	0.83-2.01
Q4	84	1.21	0.71-2.05	69	1.16	0.75-1.78
6-mo^b CMV						
Q1	45	1	Ref.	32	1	Ref.
Q2	46	1.55	0.83-2.88	36	1.11	0.63-1.96
Q3	43	1.1	0.55-2.21	36	1.23	0.68-2.21
Q4	46	0.83	0.41-1.67	31	0.75	0.41-1.40
Baseline cotinine, ng/mL						
<10	278	1	Ref.	278	1	Ref.
>25	173	0.99	0.74-1.31	173	0.85	0.68-1.05
All cotinine measures,^c ng/mL						
<10	258	1	Ref.	258	1	Ref.
>25	152	0.96	0.71-1.31	152	0.88	0.70-1.11
<10	258	1	Ref.	258	1	Ref.
>25-≤190	76	0.95	0.65-1.40	76	0.97	0.73-1.29
>190 ^d	76	0.98	0.66-1.45	76	0.8	0.59-1.08

Abbreviations: CDMS = clinically definite multiple sclerosis; CI = confidence interval; CMV = cytomegalovirus; EBNA-1 = Epstein-Barr virus nuclear antigen-1; EBV = Epstein-Barr virus; IgG = immunoglobulin G; MDMS = McDonald multiple sclerosis; Q = quartile; Ref. = reference; RR = relative risk; VCA = viral capsid antigen.

^aAdjusted for age, sex, treatment, baseline 25-hydroxyvitamin D, number of T2 lesions at baseline, body mass index, baseline Expanded Disability Status Scale score, steroid use, and onset type.

^bSixty-one participants converted to CDMS and 159 to MDMS in the first 6 months of follow-up. One hundred three participants were missing a 6-month serum sample and 14 were lost to follow-up before 6 months; all were excluded from these analyses. In addition, 2 participants had equivocal VCA IgG values and 166 were negative for CMV IgG and were excluded from the respective analysis.

^cCotinine measures: >25 ng/mL indicates active tobacco use; <10 ng/mL indicates no tobacco use over the course of the first 24 months.

^dMedian cotinine level among those with levels >25 ng/mL; indicates heavy tobacco use.

associated with conversion to MS (CDMS or MDMS) or MS activity or progression as indicated by clinical and MRI outcomes over a 5-year follow-up period. IgG antibodies to EBV-VCA and CMV also were not associated with these outcomes.

Several studies have been conducted to examine the role of EBV in MS progression. Only 3 of these focused on CIS and early MS similar to our study.²⁻⁴ Consistent with our results, among 211 participants with CIS in the SET study,² neither EBNA-1 nor VCA IgG was associated with conversion to CDMS or relapses over 2 years. However, where we found no

associations between these EBV antibodies and MRI outcomes, both were associated with an increased number of active lesions in the SET study. Only VCA was associated with EDSS progression over 2 years. In a study by Lünemann et al.³ in 147 patients with CIS, EBNA-1 IgG was not associated with conversion to CDMS or MDMS after adjustment for number of oligoclonal bands and sex, but baseline EBNA-1 IgG levels did positively correlate with number of new T2 lesions and EDSS at 1 and 5 years post onset. Similar results were reported by Farrell et al.⁴ in a small study of 50 patients with CIS followed by MRI

Table 3 Viral IgG levels at 6 months, smoking status, and risk of relapse from 6 to 60 months^a

	EBNA-1, RR (95% CI)	VCA, RR (95% CI)	CMV, RR (95% CI)	Cotinine >25 ng/mL, ^b RR (95% CI)	Cotinine >190 ng/mL, ^b RR (95% CI)
Q1	1 (ref.)	1 (ref.)	1 (ref.)	0.83 (0.47-1.45)	0.84 (0.41-1.74)
Q2	1.02 (0.53-1.96)	1.20 (0.52-2.73)	1.34 (0.60-2.99)		
Q3	1.72 (0.85-3.49)	1.03 (0.47-2.23)	1.16 (0.46-2.94)		
Q4	1.21 (0.63-2.34)	1.10 (0.50-2.42)	2.61 (0.98-6.95)		

Abbreviations: CI = confidence interval; EBNA-1 = Epstein-Barr virus nuclear antigen-1; IgG = immunoglobulin G; Q = quartile; ref. = reference; RR = relative risk; VCA = viral capsid antigen.

^a Adjusted for age, sex, treatment, baseline 25-hydroxyvitamin D, number of T2 lesions at baseline, body mass index, baseline Expanded Disability Status Scale score, steroid use, and onset type.

^b Versus cotinine <10 ng/mL.

for 3 years and clinically for 5 years. EBNA-1 IgG predicted change in EDSS score and an increase in T2 lesion volume. EBNA-1 IgG levels were higher in patients with CIS who converted to CDMS over 5 years. These positive findings, however, could be attributable to the inclusion in these studies of a few patients who did not have MS. In the study by Lünemann et al.,³ there were no MRI inclusion criteria, so that some of the patients with CIS were likely at very low risk of developing MS. Conversely, BENEFIT participants were required to have 2 or more clinically silent lesions ≥ 3 mm on a T2-weighted brain MRI scan, increasing the probability that they had MS. Only 59.2% of patients with CIS in the study by Lünemann et al.³ developed MDMS after 7 years, as compared with more than 80% of BENEFIT participants in 5 years.¹³ Because EBNA-1 IgG levels are a strong predictor of MS risk,¹⁴ the inclusion of even a small number of patients without MS, who will have no evidence of new active lesions or relapses or progression in EDSS, will cause a spurious positive association between EBNA-1 IgG levels and MS outcomes. This interpretation is consistent with the finding that studies among individuals with established MS have largely found no associations between EBV (reactivation or antibody levels) and markers of MS progression or activity.¹⁵⁻¹⁷ The use of disease-modifying therapies may also have contributed to differences across studies: all of the participants in the BENEFIT and SET² studies were treated with interferon beta, while treatment use varied in the other studies. Overall, however, the weak association between EBNA-1 IgG levels and MS prognosis stands in contrast with their strong and robust positive association with MS risk.

Two previous studies among patients with CIS on smoking and risk of conversion to CDMS found conflicting results. One study of 129 Austrian patients with CIS found a nearly 2-fold increased risk of CDMS among smokers vs nonsmokers over a 3-year observation period after adjusting for treatment, number of T2 lesions, sex, CIS onset type, and age.⁵ In contrast, and consistent with our findings,

among 194 participants with CIS in the SET study, cotinine levels >10 ng/mL were not associated with an increased risk of conversion to CDMS over 2 years.² However, they were associated with a higher gadolinium-enhancing lesion count after 2 years but not with brain atrophy. Prior studies among patients with established MS and longer disease duration have largely reported associations between smoking and MS progression including faster conversion to secondary progressive MS^{18,19} and associations with worsening clinical^{20,21} and MRI¹⁹ measures. Only one study among patients with MS has found no association between smoking and progression,²² but smoking data were collected retrospectively and many of the cohort participants either did not provide smoking data or had died and could not provide this information. It is not clear why we did not find associations between cotinine and MS progression whereas other studies consistently show that smoking is related to MS progression measures. One possibility is that our follow-up may not be long enough to observe smoking-related changes: the duration of MS in the BENEFIT participants is less than 5 years and in previous studies, the average disease duration is 9 years or more. There are also some limitations to using cotinine as a marker of tobacco use. One is that in using measures of cotinine as a proxy for smoking as opposed to self-report of smoking history, past smokers would be grouped with nonsmokers. The half-life of cotinine in blood is approximately 17 hours²³ and levels in past smokers would return to nonsmoking levels. If there are residual effects of past smoking on current MS activity or progression, we would see an attenuation of any true effect. However, one previous study found similar results on MS progression in ex-smokers and nonsmokers.²¹ Furthermore, it seems unlikely that any misclassification could fully account for the null results reported here. It is also possible that light tobacco users may be classified as nonusers, although it seems unlikely that they would go undetected because we have up to 4 measurements of cotinine and all available measurements had to be <10 ng/mL for classification

Table 4 Association^a of viral IgG levels, smoking status, and MRI measures from month 12 to 60

	6-mo measurement		
	EBNA-1	VCA	CMV
T2 lesion volume			
% change (95% CI), p value	−7.3 (−30 to 16), 0.54	−3.5 (−23 to 16), 0.33	−0.09 (−0.31 to 0.13), 0.42
Brain volume			
% change (95% CI), p value	−0.11 (−0.31 to 0.08), 0.25	−0.05 (−0.21 to 0.10), 0.50	0.001 (−0.002 to 0.004), 0.45
Cumulative no. of newly active lesions			
RR (95% CI), p value	1.06 (0.95–1.19), 0.32	0.97 (0.76–1.23), 0.76	1.00 (0.99–1.00), 0.64
			0.93 (0.64–1.34), 0.69
			0.89 (0.55–1.43), 0.63

Abbreviations: CI = confidence interval; CMV = cytomegalovirus; EBNA-1 = Epstein-Barr virus nuclear antigen-1; IgG = immunoglobulin G; RR = relative risk; VCA = viral capsid antigen.

^aAdjusted for age, sex, treatment, baseline 25-hydroxyvitamin D, number of T2 lesions at baseline, body mass index, baseline Expanded Disability Status Scale score, steroid use, and onset type.

^bVersus nonsmokers (cotinine <10 ng/mL); >25 indicates active tobacco use and <10 indicates no tobacco use over the course of the first 24 months.

in that group. Similarly, while individuals who use nicotine replacement therapies would have detectable levels of cotinine, many people use them for short periods of time, and we required all available samples (up to 4 over 24 months) to have cotinine levels >25 ng/mL for an individual to be considered a tobacco user.

The strengths of our study are the longitudinal design, large sample size (468 patients with CIS), multiple samples per patient, and systematic collection of clinical and MRI data. As mentioned above, the majority of BENEFIT participants were treated with interferon beta-1b and the results observed here may not be generalizable to patients on other therapies. Furthermore, BENEFIT participants were largely white Europeans and Canadians (98%), and whether there is no association between EBV or cotinine and MS prognosis in other populations needs study. Finally, while our study supports no direct effect of EBV or smoking on MS progression, we cannot rule out the possibility of indirect effects.

Whereas there is substantial evidence supporting a causal role for both EBV infection and cigarette smoking in the etiology of MS,¹ neither EBNA-1 IgG titers nor cotinine levels were significantly associated with MS prognosis in this large prospective investigation among patients with CIS treated with interferon beta-1b.

AUTHOR CONTRIBUTIONS

K.L. Munger: drafting/revising the manuscript for content, study concept and design, analysis and interpretation of data. K.C. Fitzgerald: drafting/revising the manuscript for content, analysis and interpretation of data. M.S. Freedman: drafting/revising the manuscript for content. H.-P. Hartung: drafting/revising the manuscript for content. D.H. Miller: drafting/revising the manuscript for content. X. Montalbán: drafting/revising the manuscript for content. G. Edan: drafting/revising the manuscript for content. F. Barkhof: drafting/revising the manuscript for content. G. Suarez: drafting/revising the manuscript for content. E.-W. Radue: drafting/revising the manuscript for content. R. Sandbrink: drafting/revising the manuscript for content. L. Kappos: drafting/revising the manuscript for content. C. Pohl: drafting/revising the manuscript for content, study concept and design. A. Ascherio: drafting/revising the manuscript for content, study concept and design, analysis and interpretation of data.

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